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Differential localization of allograft nitric oxide synthesis: comparison of liver and heart transplantation in the rat model

P. C. KUO,* E. J. ALFREY,* N. R. KRIEGER,* K. Y. ABE,* P. HUIE,† R. K. SIBLEY† & D. C. DAFOE* **Department of Surgery and †Department of Pathology, Stanford University Medical Center, Stanford, CA, USA*

SUMMARY

Nitric oxide (NO) is a free radical with a diversity of cellular origins and potential functions. Within the realm of solid organ transplantation, NO has been the focus of much attention. Discordant reports have documented both suppression and potentiation of the alloimmune response. In addition to questions regarding its functional role, little is known of the cellular origins of NO in acute rejection of vascularized allografts. To address this question, acute rejection models of rat heterotopic heart and orthotopic liver transplantation were chosen. When compared with naive controls and isografted animals, acute rejection in both heart and liver transplantation was associated with elevated systemic levels of the NO metabolite, nitrite. This was accompanied by increased graft content of iNOS protein as determined by immunoblot analysis of protein extracts. Expression of iNOS mRNA was localized with *in situ* hybridization. In both heart and liver transplantation, iNOS mRNA was found in the inflammatory infiltrate accompanying acute rejection. In addition, hepatocytes also expressed iNOS mRNA in the rejecting liver allograft. In contrast, cardiac myocytes in the rejecting heart allograft did not stain for iNOS mRNA. These results indicate that organ-specific, differential cellular expression of iNOS occurs in the acutely rejecting allograft. Transcriptional regulation of iNOS may vary among various organs according to the local cellular milieu. In addition, there may be a variable allograft specific response to acute rejection which may modify the associated immunologic biology.

INTRODUCTION

Nitric oxide (NO) is a ubiquitously produced free radical that is presumed to serve regulatory functions in virtually every mammalian organ system.[1] Three distinct NO synthase (NOS) enzymes have been identified, but one, iNOS or NOS2, is characterized by its calcium- and calmodulin-independent activity. Following induction by mediators such as interleukin-1 (IL-1), interferon-γ (IFN-γ), tumour necrosis factor (TNF) or endotoxin, iNOS is capable of sustained synthesis of high concentrations of NO. A number of *in vitro* and *ex vivo* models have demonstrated induction of iNOS with subsequent synthesis of NO in such varied cells as macrophages, hepatocytes, chondrocytes, lymphocytes, cardiac myocytes, endothelium, and Kupffer cells. However, despite identification of the *in vitro* biochemical activities of NO, questions exist regarding the functional role of NO in whole animal or organ physiology and pathophysiology. Specifically, within the realm of organ transplantation, elevated systemic levels of NO metabolites accompany the acute rejection of heart, liver and small bowel allografts in both humans and rats.[2,3] While a variety of cells within the rejecting graft are certainly capable of NO synthesis, the cellular origins of NO in this context are have not been adequately defined.

To identify the cellular origins of NO in acute rejection, the techniques of *in situ* hybridization and immunoblot analysis were utilized in rat models of heterotopic heart and orthotopic liver transplantation. In liver transplantation, iNOS mRNA was identified in both hepatocytes and portal inflammatory cells. In heart transplantation, iNOS mRNA was present in the inflammatory infiltrate but not within the cardiac myocytes. Of interest, cells, such as vascular endothelium or smooth muscle, cardiac myocytes, and biliary epithelium, which are capable of iNOS expression in other physiologic states, contained no iNOS mRNA. These results suggest that, in acute rejection, differential cellular expression of iNOS occurs that may be dependent upon the local cellular milieu and/or the particular allografted organ. Conversely, the cellular and biochemical manifestations of acute rejection may vary according to the organ of interest.

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Correspondence: Dr P. C. Kuo, Division of Transplantation Surgery, 29 South Greene St., No. 200, Baltimore, MD 21201, USA.

MATERIALS AND METHODS

Technique of liver and heart transplantation

Male rats, 250–300 g, were purchased from Harlan Sprague–Dawley (Indianapolis, IN) and housed in a virus-free, locked

animal research facility which conforms to NIH guidelines. Animals were fed rat chow, *ad libitum* and had free access to water. The DA (DA, $RT1^a$) into Lew strain combination served as the model for acute rejection (AR, $n = 4$ for heart or liver). Isografts (ISO) were performed with the Lew-to-Lew combination ($n = 4$ for heart or liver), while naive Lew rats served as additional controls (CONT, $n = 4$). No immunosuppressive therapy was administered. Unless otherwise noted, serum and tissue specimens were obtained from AR at 10-days post-transplant, and ISO at 100-days post-transplant. Orthotopic liver transplantation was performed with the Kamada technique, as previously described.[4,5] The donor liver was preserved in University of Wisconsin solution (DuPont Pharmaceuticals, Waukegen, IL). A cuff (Intramedic polyethylene tubing, Thomas Scientific, Swedes Boro, NJ) was placed on the portal vein and infra-hepatic vena cava. After the recipient hepatectomy, the supra-hepatic vena cava was anastomosed with a 7–0 vascular suture. The portal vein and infrahepatic vena cava cuff was then secured with a free ligature. The bile duct was internally stented with a polyethylene stent. The portal vein was clamped for less than 25 min in all animals. In all treatment groups, survival following orthotopic liver transplant (OLT) was >80%. Heart allografts were placed heterotopically into the abdomen similar to that described by Ono.[6] Briefly, the donor rat was heparinized then killed. The heart was cooled with iced saline and removed. The heart allograft was then revascularized by anastomosing the donor aorta to recipient infrarenal aorta and pulmonary artery to the vena cava.

In situ *hybridization and histologic analysis*[7]

Liver tissue was obtained from animals, immediately embedded in Optimal Cutting Temperature compound (Miles Inc., Elkhart, IN) and snap frozen in liquid nitrogen. Tissues were stored at −70° until use. Tissues were cut into 6-μ thick sections at −20° and mounted onto RNAse free, uncoated glass slides. Sections were immediately fixed for 10 min in 4% paraformaldehyde in phosphate buffered saline (PBS) with 5 mM magnesium chloride, pH 7·3, and dehydrated with a graded series of ethanol/water solutions. Tissue sections were then prehybridized with an RNAse-free 40% formamide/transfer RNA/Denhardt's hybridization solution (FTD; Denhardt's reagent, Boehringer Mannheim, Indianapolis, IN) for 1 hr at 42° in a chamber with 100% humidity. Slides were hybridized with a 1·5 ng/ml solution (in FTD) of 3′ end-labelled digoxigenylated oligomer (30mer) DNA, sealed with RNAse-free coverslips, and incubated overnight at 42° in a chamber with 100% humidity. Based upon the published murine macrophage iNOS cDNA sequence,[8] three distinct oligonucleotide iNOS probes in an anti-sense orientation were designed with an GC:AT ratio between 0·7–0·8 to standardize hybridization kinetics: AAGTCCAGCCGCACCACCCTCCTCGTTCAG, CACGGACGAGACGGATAGGCAGAGATTGG, and ACCAAAATGGCTCC CCGCAGCTCCTCACT. Three distinct sense probes for iNOS mRNA served as negative controls: CTGAACGAGGAGGGTGGTGCGGCTGGACTT, CCAATCTCTGCCTATCCGTCTCGTCCGTG, and AGTGAGGAGCTGCGGGGAGCCATTTTGGT. Confirmation of oligonucleotide antisense probe specificity for rat iNOS mRNA was determined with Northern blot analysis (data not shown). A polythymidylate nucleotide probe served as a positive control.. All probes were obtained from Operon Technologies Inc. (Alameda, CA). Hybridizations with probe-free FTD were performed as an additional control to determine endogenous signal. Control and experimental tissues were hybridized concurrently. All probes were end-labelled before use with the digoxigenin-labelling kit (Boehringer Mannheim) and stored at −20°. Sections were washed for 30 min each in RNAse-free 5× saline sodium citrate (SSC), 2× SSC, 1× SSC, 0·5× SSC and 0·25× SSC. Slides were then blocked with 2% normal sheep serum in 0·1 M Tris-buffered saline, pH 7·5, for 1 hr at room temperature and incubated with sheep serum-blocked 0·5% anti-digoxigenin sheep Fab fragments conjugated with alkaline phosphatase. Sections were developed with McGadey's reagent [nitro-blue tetrazolium (NBT) + 5-bromo-4-chloro-3-indoyl-2-phosphate (BCIP) in 0·1 M Tris-buffered saline, pH 9·5, +10 mM levamisole] for 10 min at room temperature. Developing reactions were stopped with tap water and the sections were secured with a coverslip. Tissues were visualized on a Leica microgrid and scored in a blinded fashion. Positive staining was defined as a cell with a dark purple-blue cytoplasm. Tissues stained with poly-thymidylate probes or FTD alone were analysed only for the presence or absence of signal. Liver and heart tissues were also fixed in 10% formalin and stained with haematoxylin and eosin (H and E) for visualization by light microscopy.

Immunoblot analysis

Immediately following retrieval, liver or heart tissue was sonicated and washed three times in PBS and incubated with boiling 2× non-reducing electrophoresis sample buffer for 2 min. Separation was performed on a 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and then electrotransferred to a polyester-supported nitrocellulose membrane for 90 min at 150 mA. The membrane was blocked overnight at 4° in Tris-buffered saline (10 mM Tris-HCl at pH 7·5, 150 mM NaCl) containing 3% bovine serum albumin (BSA). Blocked membranes were incubated with iNOS antibody (monoclonal primary anti-murine macrophage iNOS antibody from Transduction Laboratories, Lexington, KY), washed three times in Tris-buffered saline/0·1% Tween, and incubated with biotinylated sheep anti-murine IgG (Amersham Inc., Arlington Heights, IL) for 1 hr. After washing again three times, membranes were then incubated with strepavidin-horse radish peroxidase conjugate. Following washing, bound antibodies were detected by the enhanced chemiluminescence (ECL) detection system (Amersham, Arlington Heights, IL). Blots were scanned with a computerized laser densitometer (Hoeffer Scientific Instruments, San Francisco, CA) and the area under the curve normalized to the murine macrophage iNOS enzyme standard.

Measurement of NO metabolites

Serum concentration of NO was quantified by measurement of the NO metabolite, nitrite, using a modified version of the technique of Snell & Snell.[9] Following deproteinization with 0·5 M NaOH and 10% $ZnSO_4$, serum samples were mixed with 1% sulfanilamide in 0·5 N HCl (50%; v/v). After a 5-min incubation at room temperature, an equal volume of 0·02% naphthylenediamine was added. Following incubation at room temperature for 10 min, absorbance at 570 nm was compared with that of an $NaNO_2$ standard.

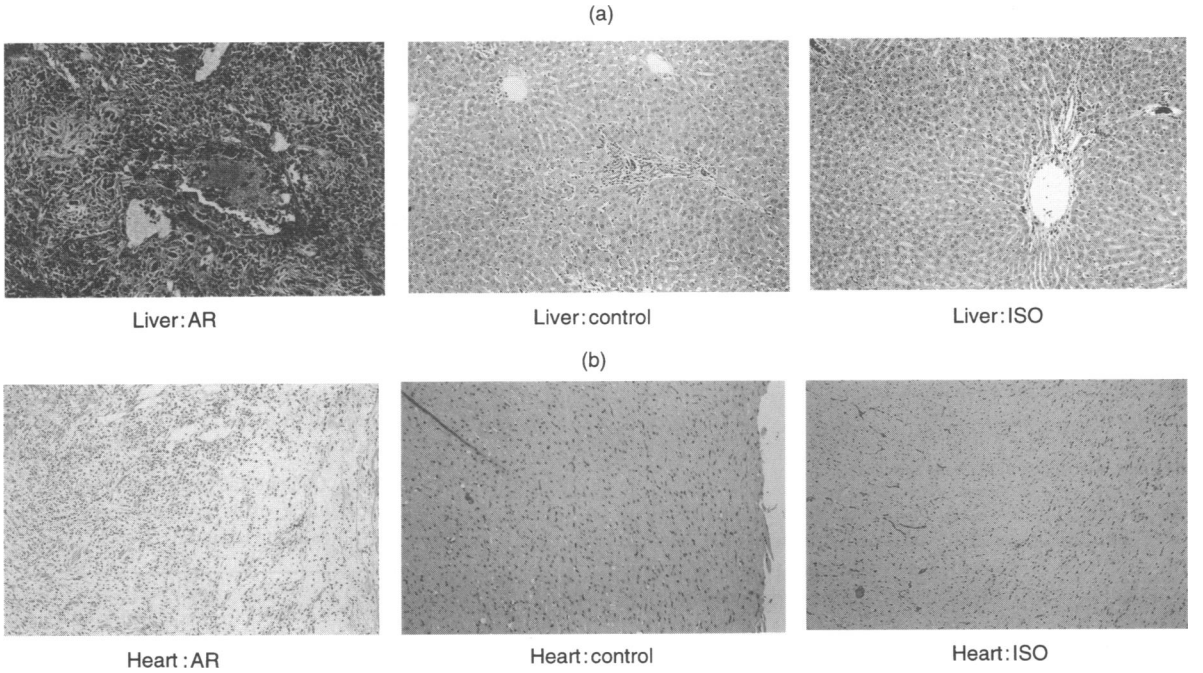

Figure 1. (a) Representative haematoxylin and eosin stains of tissue sections from control, isograft (ISO) and acutely rejecting (AR) rat orthotopic liver transplants. (b) Representative hematoxylin and eosin stains of tissue sections from control, isograft (ISO) and acutely rejecting (AR) rat heterotopic heart transplants.

Measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (T Bili)

Serum levels of AST, ALT and T Bili were measured by the Gemstar automated colorimetric technique (Schiapparelli Biosystems Inc., Fairfield, NJ).

Statistical methods

All values are presented as the mean ± standard error (SE) of the mean (SEM) of three or four animals or experiments. Data were analysed with the two-tailed paired or unpaired Student's *t*-test, as appropriate. *P* values less than 0·05 were considered to be statistically significant.

RESULTS

Histology and function of liver and heart grafts in AR, ISO and CONT animals

Tissue sections of heart and liver grafts from AR, ISO and CONT animals were examined (Fig. 1). In the context of OLT, ISO allografts did not exhibit any evidence of rejection and were not found to be significantly different than that of CONT. However, in AR, the liver allografts were found to have predominantly mononuclear portal infiltrates with inflammatory cell linkage of the triads, lobular necrosis and hepatocyte dropout, and scattered regions of vascular thrombosis. These findings are consistent with severe acute rejection of the liver allograft. Biochemically, in CONT and ISO animals, the AST, ALT and T Bili were 77 ± 6·0 U/l and 92 ± 21 U/l, 44 ± 4·6 U/l and 43 ± 17 U/l, and 0·3 ± 0·1 mg/dl and 0·4 ± 0·1 mg/dl respectively. In contrast, in AR animals, the AST, ALT and T Bili were 632 ± 57 U/l, 183 ± 39 U/l and 4·9 ± 0·8 mg/dl (all $P < 0·01$ versus CONT or ISO).

Heart allografts from ISO and CONT animals were found to have normal histologic architecture without evidence of rejection. In AR animals, the heart allografts exhibited marked interstitial inflammatory infiltrates with myocyte damage. Myocyte necrosis and interstitial oedema were multifocal. In areas of higher infiltrate density, interstitial haemorrhage was evident. Overall, the histologic picture was that of severe acute rejection. Graft function as determined by palpation of the heterotopic heart allograft was normal in both ISO and CONT animals. In contrast, AR animals uniformly had substantially diminished heart allograft function.

Serum NO and allograft iNOS protein levels

Serum samples from the various treatment groups were analysed for the presence of the NO metabolite, nitrite (Table 1). Minimal amounts of nitrite were present in the CONT or ISO groups for heart or liver transplants. In contrast, AR was associated with a four- to six-fold elevation in serum NO levels in both heart and liver allografts animals ($P < 0·01$ versus CONT or ISO for both heart and liver transplants). Immunoblot analysis of cellular protein extracts from heart and liver grafts was performed to determine the relative quantities of iNOS protein expression within the respective allografts (Fig. 2). Heart or liver allografts in CONT and ISO animals did not express any detectable iNOS. In contrast, in AR animals, heart allografts and liver allografts were found to express significant levels of iNOS protein. Densitometric analysis of heart-AR and liver-AR animals found iNOS protein levels, relative to a murine macrophage iNOS control, to be 3·7 ± 0·7 and 4·2 ± 0·8, respectively (P = NS). These results suggest that elevated systemic NO levels are, in part, derived from iNOS enzyme activity in the acutely rejecting heart or liver allograft.

Table 1. Systemic nitrite levels in heart and liver allografted animals

	Heart	Liver
Control	7·6 ± 0·9	8·6 ± 1·2
Isograft	8·7 ± 1·1	9·2 ± 1·2
Acute rejection	45·2 ± 2·7*	56·0 ± 3·3*

* $P < 0.01$ acute rejection versis control or isograft.

Data are presented as mean ± SEM of four animals within each group.

Nitrite levels are expressed as μM.

Table 2. Allograft expression of inducible nitric oxide synthase mRNA

	Heart	Liver
Control	0	0
Isograft	2·0 ± 0·5	1·5 ± 0·7
Acute rejection	89 ± 12*	95 ± 15*

* $P < 0.01$ acute rejection versus control or isograft.

Data are presented as mean ± SEM of four animals within each group.

Values expressed as number of positively stained cells per square mm of tissue.

Table 3. Cell-specific expression of inducible nitric oxide synthase mrna in acute rejection

	Heart	Liver
PC	1·1 ± 1·0	76 ± 5·0†
VC	2·3 ± 1·1	1·3 ± 1·2
IC	89 ± 2·5*	93 ± 3·2*

* $P < 0.01$ IC versus PC or VC.

† $P < 0.01$ liver versus heart.

Data are presented as mean ± SEM of four animals within the heart or liver group.

Values expressed as % of labelled cells per 100 PC, VC or IC.

Myocytes and hepatocytes were classified as parenchymal cells (PC); vascular endothelium, vascular smooth muscle and endocardium were classified as vascular cells (VC). Inflammatory cells (IC) formed a third class.

In situ hybridization for iNOS mRNA

Positively stained cells in heart and liver allografts in AR, ISO and CONT animals were counted and expressed per mm^2 of tissue (Fig. 3, Table 2). In both heart and liver transplantation, AR was associated with significantly increased iNOS mRNA expression ($P < 0.01$ AR versus CONT and ISO). The extent of staining was 40- to 60-fold greater in AR in comparison to the other treatment groups. The AR specimens were then further analysed to determine the particular cell types expressing iNOS mRNA (Table 3). Myocytes and hepatocytes were classified as parenchymal cells (PC); vascular endothelium, vascular smooth muscle and endocardium were classified as vascular cells (VC). Inflammatory cells (IC) formed the third and final class. Positively stained cells were expressed as the percentage of labelled cells per 100 PC, VC or IC counted. In heart transplants, the vast majority of iNOS positive cells were IC; 89% of IC stained positively for iNOS mRNA. Minimal iNOS mRNA was present in myocytes, vascular endothelium or vascular smooth muscle. In contrast, in liver transplants, both IC and PC (i.e. hepatocytes) stained positively for iNOS mRNA. Among IC, 93% expressed iNOS mRNA, while among the hepatocytes, 76% contained iNOS mRNA. Again, like that of the heart transplant animals, VC in liver transplant animals expressed minimal iNOS mRNA.

DISCUSSION

NO is a ubiquitously synthesized free radical with a diversity of molecular origins, targets and actions. While a variety of biochemical actions have been identified, the function and origin of NO in physiologic states have not been adequately defined. Specifically, the role of NO and site of synthesis in the immunologic states following organ transplantation are largely unknown.

Previous work on the function of NO in the alloimmune response has resulted in contrasting results. Langrehr and

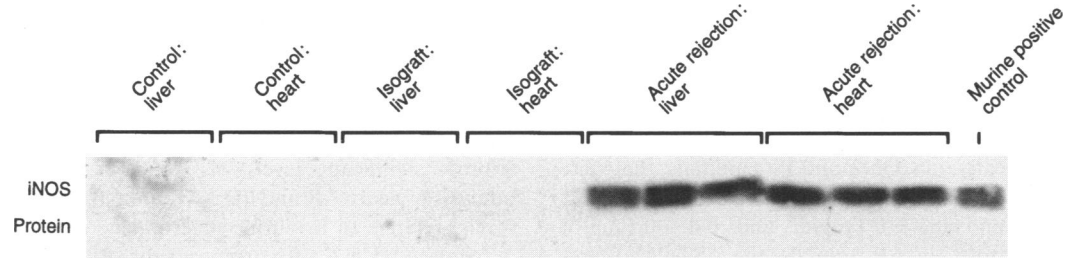

Figure 2. Immunoblot analysis of iNOS protein expression in crude cellular protein extracts from individual rat heterotopic heart and orthotopic liver transplants. Purified murine macrophage iNOS protein served as a control for both the molecular weight of the iNOS enzyme and specificity of the monoclonal iNOS antibody.

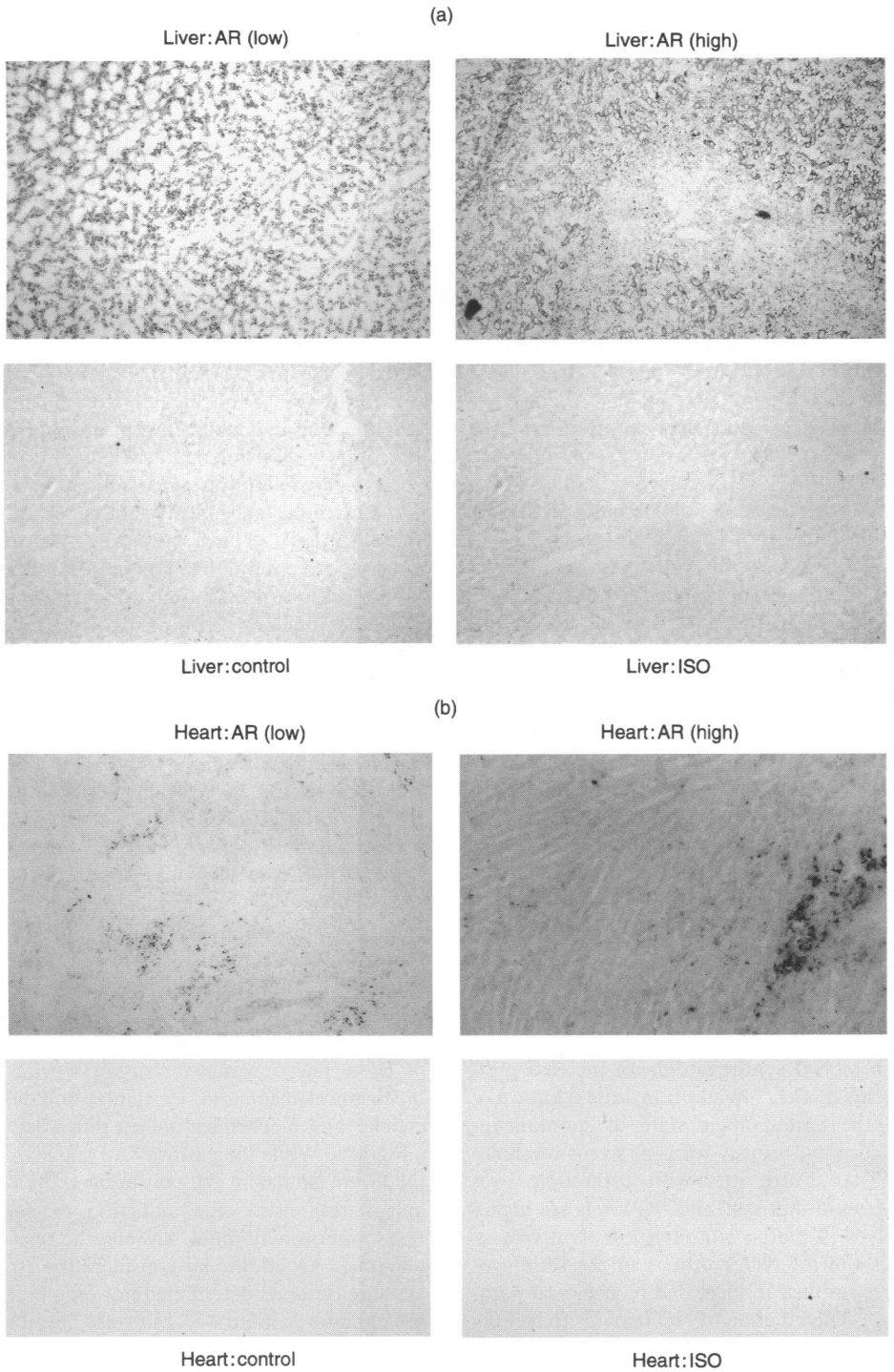

Figure 3. (a) Representative *in situ* hybridization for iNOS mRNA in tissue sections from control, isograft (ISO) and acutely rejecting (AR) rat orthotopic liver transplants. Low, 19× magnification; High, 30·4× magnification. (b) Representative *in situ* hybridization for iNOS mRNA in tissue sections from control, isograft (ISO) and acutely rejecting (AR) rat heterotopic heart transplants. Low, 19× magnification; High, 30·4× magnification.

co-workers have demonstrated that inhibition of NO synthesis potentiates the proliferative and cytolytic T-cell activity in murine and rat mixed lymphocyte cultures.[10,11] In the sponge matrix model, *in vitro* restimulation of allograft infiltrating cells with the sensitizing alloantigen resulted in increased NO synthesis and concomitant inhibition of cytotoxic T-lymphocyte proliferation. In addition, the same authors have postulated that macrophage antigen-presentation function may be impaired as a result of NO-mediated alterations in cytoplasmic pH regulation. Others have reported that NO inhibits splenic lymphocyte proliferation without altering IL-2 production.[12] However, other investigators have reported

contrasting results. Gregory and co-workers have demonstrated that inhibition of NO synthesis results in suppression of *in vitro* T-lymphocyte proliferation.[13] In a murine model, Roland *et al.* have reported that NO synthesis impacts negatively on portal induction of tolerance.[14] With regard to these discordant reports, the observations of Rubbo and Lipton may be pertinent.[15,16] Rubbo and co-workers addressed the neuroprotective and neurotoxic effect of congeners of NO. They report that the redox versatility of NO allows its interconversion from neuroprotective to neurotoxic species by a change in the ambient redox milieu.[15] Lipton *et al.* have demonstrated that the differential effects of NO as a protective or toxic agent in oxidative stress occur as a result of relative concentrations of NO and superoxide.[16] These results indicate that the relative concentration of NO, in part, dictates its seemingly contradictory effects and may contribute to the seemingly discordant observations regarding its immunologic functions.

Another potentially relevant immunologic function may be the role of NO as an endogenous modulator of leucocyte adhesion.[17–19] Kubes and colleagues report that inhibition of NO synthesis results in superoxide and mast cell-dependent adhesion in a rat mesentery model.[17] In other studies by our group, cytokine-mediated hepatocyte synthesis of NO is protective in *in vitro* models of oxidative injury.[20,21] Furthermore, in the setting of cytokine stimulation, NO synthesis by the hepatocyte is involved in the transcriptional regulation of gamma-glutamylcysteine synthetase, the rate-limiting enzyme for glutathione production (unpublished observations). While a number of potential mechanisms and functions have been identified for NO in the alloimmune response, its role remains the subject of controversy.

Two recent reports addressed the potential *in vivo* function of NO in rodent heterotopic heart models of acute rejection. Worrall and co-workers found that inhibition of iNOS function ameliorated acute rejection with prolonged graft survival, improved graft contractile function and reduction in the histologic grade of rejection.[22] In contrast, Bastian *et al.* found that inhibition of NO synthesis did not increase graft survival time in a murine model.[23] In addition, little is known of the role of NO in the immunologic state of spontaneous tolerance.[23] Using the spontaneously tolerant strain combination of Lewis-to-Wistar Furth rats with orthotopic liver transplants, we have found that systemic NO levels are higher than that of control or isografts, although less than that of acute rejectors. Inhibition of NO synthesis in the setting of spontaneous tolerance resulted in increased biochemical parameters of liver injury.[24] The function of NO remains to be fully clarified. Protective or injurious effects of NO may again depend upon the relative local concentrations of NO and accompanying biologic modifiers such as IL-1, TNF or IFN-γ.

An observation that has been established and confirmed is the association between elevated systemic levels of NO and acute rejection. Langrehr and colleagues have reported that elevated levels of NO accompany acute rejection in rat models of heart, liver and small bowel transplantation. Treatment with cyclosporine or tacrolimus resulted in inhibition of NO synthesis. The authors suggest that NO metabolites may serve as markers for acute rejection.[2] In human recipients of orthotopic liver transplants, plasma NO levels increased in the setting of acute rejection, correlated with rejection severity, and decreased following treatment with corticosteroids.[3] Organ-specific localization of the site for elevated NO synthesis has also been performed. In the rat heterotopic heart model of acute rejection, Lancaster *et al.* found that the allograft was a source for increased NO synthesis.[25] Using the technique of electron paramagnetic resonance spectroscopy, iron–nitrosyl species were found in the allografted heart but not in the spleen, liver or lung.

Yet another question is the cellular origin of NO that accompanies acute rejection. In work by Yang *et al.* and Worrall *et al.* using the rat heterotopic heart transplant model, acute rejection was associated with increased allograft production of NO.[22,26] However, the cellular origin of NO differed between the two studies. Using immunocytochemical staining for iNOS, Yang and co-workers found that myocytes, endothelium and infiltrating macrophages expressed iNOS.[26] In contrast, Worrall and colleagues found that iNOS was present only in infiltrating inflammatory cells without evidence for immunohistochemical staining in endothelium, vascular smooth muscle or myocytes.[22] In a rat model of acute renal allograft rejection, iNOS has been localized to the infiltrating mononuclear cells without evidence for expression in renal tubular epithelium.[27] Localization of iNOS in the setting of orthotopic liver transplantation has not been previously performed. In our study, acute rejection of heart or liver allografts was associated with increased systemic levels of the NO metabolite, nitrite. Immunoblot analysis localized one site of NO synthesis, among others, to the rejecting allograft. Furthermore, *in situ* hybridization was performed to localize iNOS mRNA expression. In heart transplants, iNOS mRNA was present only in the inflammatory mononuclear infiltrate. In contrast, iNOS mRNA was present in both hepatocytes and inflammatory cells in rejecting liver allografts. This differential cellular expression of iNOS mRNA is significant in light of the multitude of cells that are capable of transcription of the iNOS gene: myocytes, hepatocytes, macrophages, biliary ductal epithelium, vascular smooth muscle cells, and endothelium, among others.[1,28] These observations suggest that the process of acute rejection may be organ specific with respect to its biochemical mediators. Certainly, our data suggests that the cellular and biochemical milieu differ between heart and liver allografts. While the pathway for transcriptional regulation of iNOS are similar in the two organs, the relative quantities of agents responsible for induction or suppression of iNOS transcription may differ between the rejecting heart and liver allograft. An additional consideration is that the process of acute rejection is determined not only by the properties of the inflammatory infiltrate, but also by the response of the parenchymal cells within the specific graft. This allograft response may be organ specific as demonstrated by our observations.

In summary, the functional role of allograft NO synthesis remains the subject of controversy with contrasting results in various models of acute rejection and tolerance. In addition, the cellular location of NO synthesis is largely unknown. To address this question, we utilized the technique of *in situ* hybridization to localize iNOS mRNA acute-rejection models of rat heterotopic heart and orthotopic liver transplantation. In both instances, iNOS mRNA was present in the inflammatory infiltrates. Hepatocytes were also noted to strongly express iNOS mRNA in the rejecting liver allografts; in contrast,

cardiac myocytes did not stain for iNOS. These hybridization results correlated with local levels of iNOS protein and systemic NO metabolites. This differential expression of iNOS within the allograft suggests that transcriptional regulation of the iNOS gene may be organ dependent. In addition, our observations indicate that the responses of parenchymal cells to acute rejection vary among allografted organs and as a result, may modify the overall biological consequences associated with an alloimmune response.

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